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13. ABSTRACT *(Maximum 200 Words)* Abundant data implicates maximal microvessel density in breast and many other solid tumors as being a strong prognostic indicator. These data are gained by quantitating the number of microvessels in "hot spots" of high-density tumor vasculature, implying that such hot spots have functional significance in the process of metastasis. We proposed that gene expression in the area of the microvascular hot spots is different from elsewhere in the tumor and that genes upregulated in these areas may be functioning to promote metastasis. We have produced experimental tumors in mice and harvested them so as to preserve spatial relationships within the tumor. Microvessel densities were quantitated in several areas of each tumor. Pulmonary metastases were also quantitated and correlated with the maximal microvessel density for each tumor. Areas of tumor adjacent to the microvascular hot spots in the most metastatic tumors were microdissected along with similar sized spots not associated with hot spots. RNA was extracted from these microdissected samples and used to synthesize cDNA. Microarray analysis will be used to discover genes differentially expressed in areas adjacent to hot spots compared with areas not associated with hot spots. It is hoped that gene expression important in the process of metastasis or extravasation will be discovered.

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FOREWORD

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Sandra W. McLeskey 7/12/00

PI - Signature Date

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INTRODUCTION

We proposed a scheme to discover metastasis- or angiogenesis-promoting genes which are preferentially expressed in areas adjacent to microvessel hot spots in xenograft tumors produced by breast cancer cells injected into the mammary fat pads of nude mice. During the funding period, we have produced the xenograft tumors, quantitated metastasis and microvessel density, and correlated the two. We have identified specific xenograft tumors with the highest number of metastases and microdissected the tumor cells adjacent to the "hottest spot" in the tumor as well as tumor cells in control areas not associated with microvascular hot spots. These microdissected tumor cells were utilized to synthesize cDNA, which has been preserved at –70°. We have explored ways of amplifying cDNA from microdissected cells in order to use microarray technology for gene discovery. Over the next year, we will use institutional funding to amplify these stored cDNAs and apply them to microarrays, looking for genes consistently upregulated in the areas adjacent to the hottest spots of microvascular when compared with the control microdissected areas not associated with hot spots.

The PI on this project, Dr. Sandra McLeskey, is moving her laboratory to the University of Maryland Baltimore as of July 1, 2000. This institution has funds (DRIF funding) available for investigators to conduct research which does not have extramural funding. These funds will be available to complete this project.

BODY

Aim 1. We will identify critical microenvironments in the tumors produced by FGF-1 transfected MCF-7 cells in nude mice by sensitively and accurately correlating the degree of metastasis in the lungs and lymph nodes with maximal microvessel density in the hottest spot in each tumor.

As mentioned in the two yearly reports, we produced single xenograft tumors in each of 30 nude mice with FGF-1 transfected MCF-7 breast cancer cells [1] cotransfected with *lacZ*, which enables sensitive detection of micrometastases by X-gal staining. We sectioned the tumors completely in an orderly fashion so as to know from which area a given section was obtained. We then used image analysis to quantitate the pulmonary micrometastases as revealed by X-gal staining to determine the most metastatic tumors. Microvessel density, as revealed by PECAM-1 immunohistochemistry [2] in representative sections from each tumor was quantitated with a Chalkley graticule [3]. By examining multiple sections from each tumor, we were able to find an area of maximal microvessel density for the whole tumor. This area was deemed the "hottest spot" of microvasculature and its Chalkley score was used in subsequent correlations with metastasis.

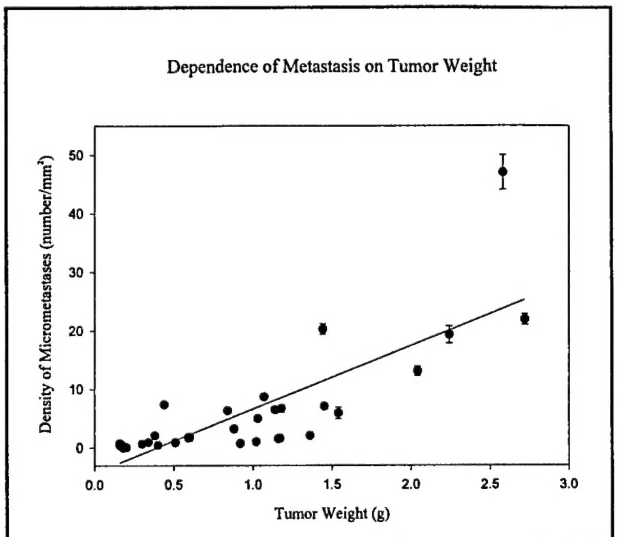


Figure 1. Density of pulmonary micrometastases correlates with tumor weight. Linear regression performed on a logarithmic transformation of density of micrometastases vs tumor weight showed the regression line to be highly significant ($p<0.001$).

We were able to confirm our previous findings [4;5] that density of micrometastasis correlates well with tumor size (Figure 1). In addition, we saw a rough correlation of microvessel density with degree of micrometastasis (Figure 2). These findings were reported in previous years.

During this past year, we have used laser capture microdissection (LCM) to microdissect areas of tumor adjacent to the "hottest spots" of microvasculature, extracted RNA, and synthesized cDNA. Control spots not associated with microvessels from the edge and center of the tumor were also harvested. The methods used for RNA extraction and cDNA synthesis were reported in previous years. Briefly, we resuspended the microdissected tissue in buffer and extracted the RNA using the Qiagen RNEasy kit and precipitated it. First strand cDNA with an appended T7 RNA polymerase promoter was synthesized using Superscript II and a template switching oligonucleotide encoding the T7 promoter. (Template switching was described in previous reports and is also described below.) This cDNA was precipitated and preserved at -70°. It will be used to synthesize aRNA for application to microarrays in connection with aim 2.

Aim 2. We will analyze differential gene expression in tumor cells in the area of hot spots by microdissection followed by differential display PCR.

Initially we proposed to use differential display PCR to examine gene expression in areas of tumor adjacent to microvessel hot spots. However, as we subsequently gained experience with the amplified fragment length polymorphism (AFLP) technique [6] in connection with another project in our laboratory, we planned to substitute this technique for differential display. However, at present, we feel it would be wasteful of time and money to use any technique other than a microarray method for examination of differential gene expression. The University of Maryland Baltimore has a microarray core facility which we will be using for this project. We will most likely use custom arrays which incorporate cDNAs associated with motility, proteolytic capability, and angiogenesis. However, we will also include other genes which might be associated with proliferation or malignancy. With this technique, we will be able to screen large numbers of genes which might be upregulated in connection with angiogenesis or metastasis.

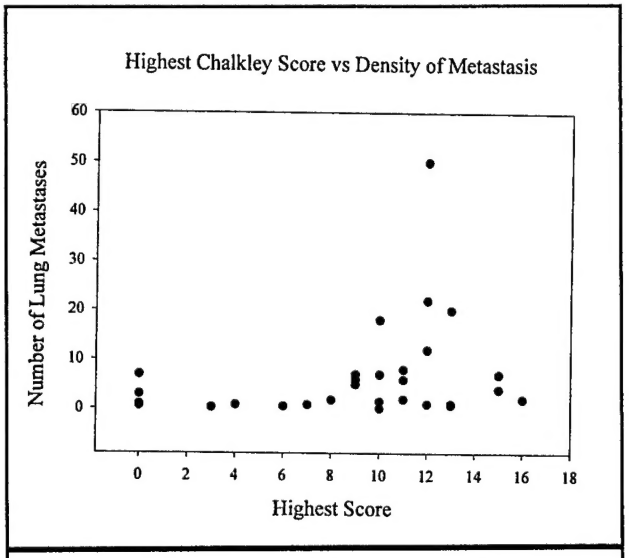


Figure 2. Relationship of lung metastases and Chalkley score. The area of maximal microvessel density for each tumor was quantified with a Chalkley graticule. Lung metastases were quantified by image analysis.

As outlined in the yearly reports, we have been exploring methods of amplification of RNA or cDNA obtained from microdissected cells so that there would be enough to use for differential cloning efforts. These efforts are particular pertinent to microarray technology, as 100 ng total RNA are necessary for application to most microarrays. Amplification of messenger RNA obtained from an approximately 30 μm microdissected spot is necessary but the method of amplification must preserve proportionality of the various mRNA species to be useful for analysis of differential gene expression. As mentioned in previous reports, there are two methods of amplification which could be used: amplified RNA (aRNA) or the polymerase chain reaction (PCR). Each method has its advantages and disadvantages, and we have been exploring each separately and both of them combined as possible methods to be used in this project.

A PCR-based method of amplification is employed by Clontech SMART™ cDNA kit. A manufactured sequence is appended to the 5' end of the first strand by using a poly-dT primer with a known sequence 5' of the poly-dT tract. A reverse transcriptase (RT) is used which appends several deoxycytidines to the 3'end of the newly synthesized first strand. If an oligonucleotide with several deoxyguanines at its 5' end is included in the reaction, this oligonucleotide will base-pair with the terminal dCs at the end of the 1st strand, and the RT will then copy the 5' sequences of the oligonucleotide, appending them to the 3' end of the 1st strand. In this way, a manufactured "tag" is appended to the 3' end of the 1st strand. Thus, known sequences are appended to each end of the 1st strand cDNA, which are then used to prime a long-distance PCR reaction under carefully controlled conditions (Figure 3). This method has been shown with Northern blots to preserve

proportional representation of particular mRNAs in the population [7]. However, as microarray technology is more sensitive than Northern blots, we felt it was important to subject this method more stringent testing, below.

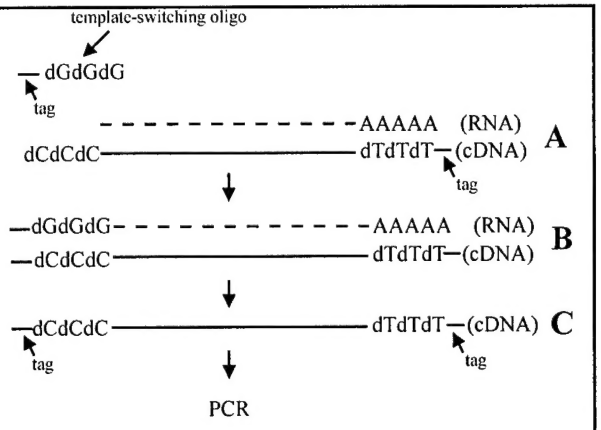


Figure 3. Diagram of template switching. In **A**, 1st strand cDNA synthesis is primed by an oligo-dT primer with a manufactured sequence (tag) appended to the 5' end. The MMLV reverse transcriptase copies the RNA strand (dashed line) to the end, whereupon it adds several terminal dC residues to the cDNA (solid line). In **B**, the template-switching oligonucleotide with several 3' dG residues has base-paired with the terminal dC residues on the 1st strand cDNA, and the reverse transcriptase has continued to copy a manufactured sequence (tag) at the 3' end of the oligonucleotide, appending it to the 3' end of the 1st strand cDNA. In **C**, the resulting 1st strand cDNA has manufactured sequences (tags) appended to both ends which can be used to amplify it by PCR.

The conventional aRNA technique involves 1st strand cDNA synthesis with a poly-dT primer which has a T7 RNA polymerase promoter appended (Figure 4). This appends a T7 RNA polymerase promoter site to the 3' end of the double stranded cDNA which produces antisense-oriented aRNA. We have obtained longer cDNA by combining this technique with template switching to append the T7 RNA polymerase promoter to the 3' end of the 1st strand cDNA (Figure 5). This results in a T7 RNA polymerase promoter at the 5' end of the double-stranded cDNA. When incubated with T7 RNA polymerase, this will produce sense-oriented aRNA which is essentially identical to the original mRNA (Figure 5). This sense RNA can be used in any way mRNA could be, but it can also serve as template for second-round of cDNA and subsequent second-round aRNA synthesis. Since each round of cDNA/aRNA synthesis produces an amplification of approximately1000-fold, two tandem amplifications will be on the order of one million-fold, an amplification comparable to that of PCR.

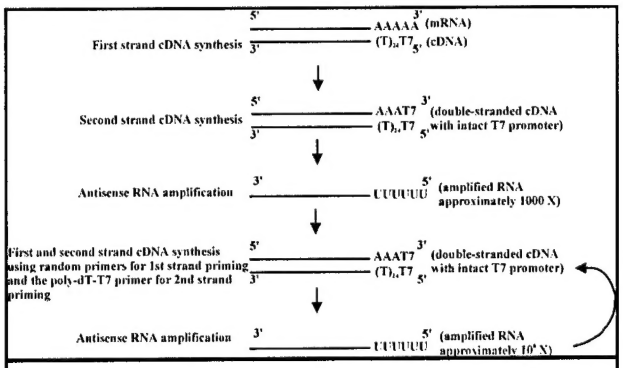


Figure 4. Scheme for conventional aRNA synthesis. 1st strand cDNA synthesis is primed with a poly-dT primer with an appended T7 RNA polymerase promoter site. Following second strand synthesis by hairpin priming, clipping and polishing, antisense-oriented aRNA is synthesized with T7 RNA polymerase. This product can be used to synthesize 2nd round cDNA with a random primed 1st strand and 2nd strand synthesis primed with the same poly-dT-T7 primer as was used to prime the 1st round 1st strand. 2nd round antisense aRNA can then be synthesized from the 2nd round cDNA.

In collaboration with Dr. Yan Su of Georgetown, we compared the fidelity of the two methods of amplification (Clontech SMART™ cDNA *versus* aRNA) with microarrays. Using 1ng total RNA from a breast tumor cell line, we synthesized cDNA by the Clontech SMART™ cDNA method or by our new aRNA method which involves template switching. Both first and second round aRNA were produced. The cDNA was radiolabeled by primer extension and the aRNA and source total RNA were radiolabeled by reverse transcription. The labeled products were hybridized to 1000 cDNAs arrayed on a filter (Research Genetics). Figure 6 depicts the pattern of hybridization obtained for the source total RNA, and for aRNA synthesized from first-round cDNA, aRNA synthesized from second-round cDNA, and cDNA synthesized with the SMART™ cDNA synthesis kit. Although the background and signal strength is different on each filter, the patterns of positive hybridizations on the filters appear by eye to be very similar. However, when

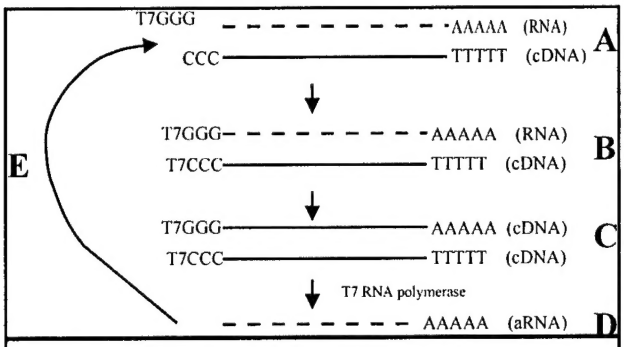
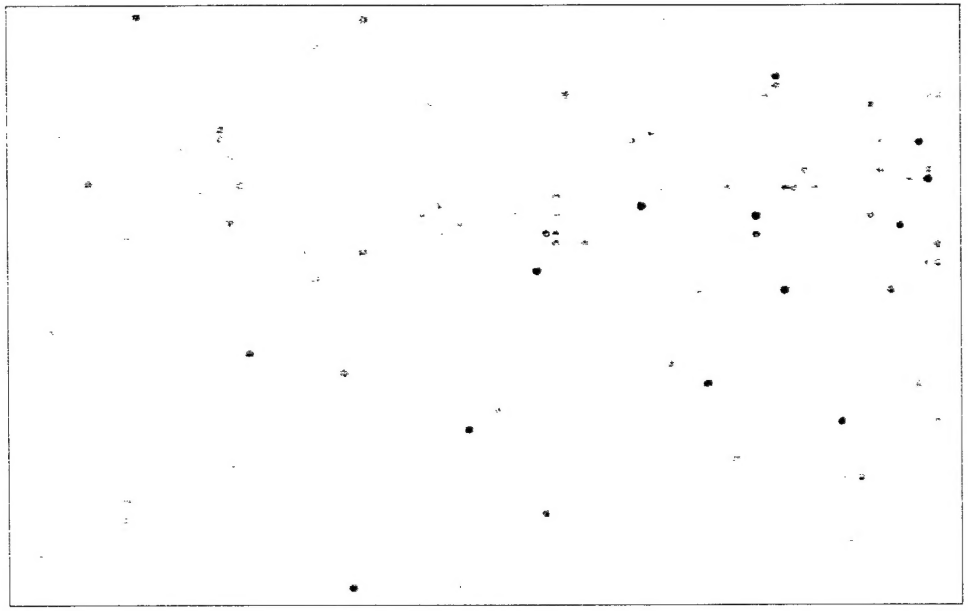


Figure 5. Combination of aRNA and template switching. A T7 RNA polymerase promoter is appended to the 3' end of the 1st strand cDNA by template switching (A and B). 2nd strand cDNA synthesis is primed by the template-switching oligonucleotide (C) and sense-oriented aRNA is produced (D). This aRNA can be used as template for 2nd round cDNA synthesis (E), followed by 2nd round aRNA synthesis.

the filters were subjected to densitometry, we found that none of the amplification methods were completely faithful. We are currently in the process of using the microarray analysis software to determine ways of handling these data so as to standardize the signals between the different amplification products. At that point, we will be in a position to determine the degree of skew introduced by each amplification method and to determine which one produces the least skew. We can also determine parameters to allow for the skew, allowing us to apply these parameters to microdissected specimens amplified by the particular method we choose. In this way, when we apply the cDNA obtained from the specimens generated in aim 1 of this project, we will be able to discriminate truly differentially expressed genes from those which appear to be differentially expressed because of skew introduced by the amplification method.



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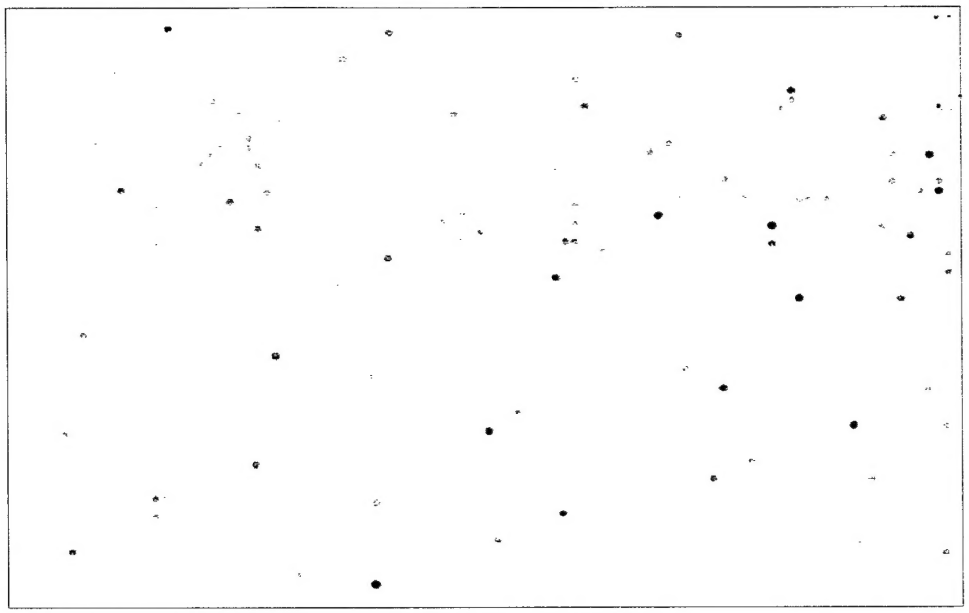
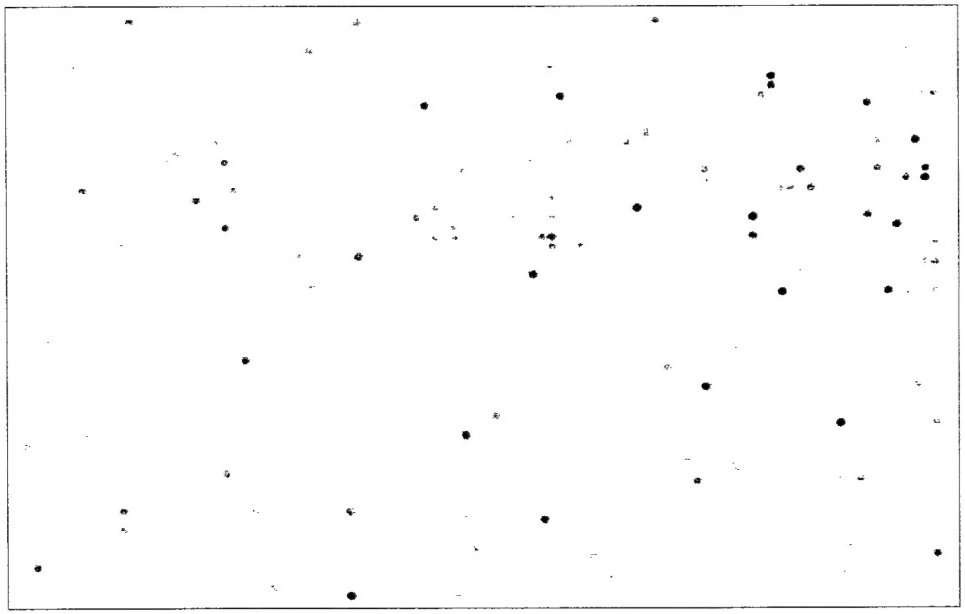
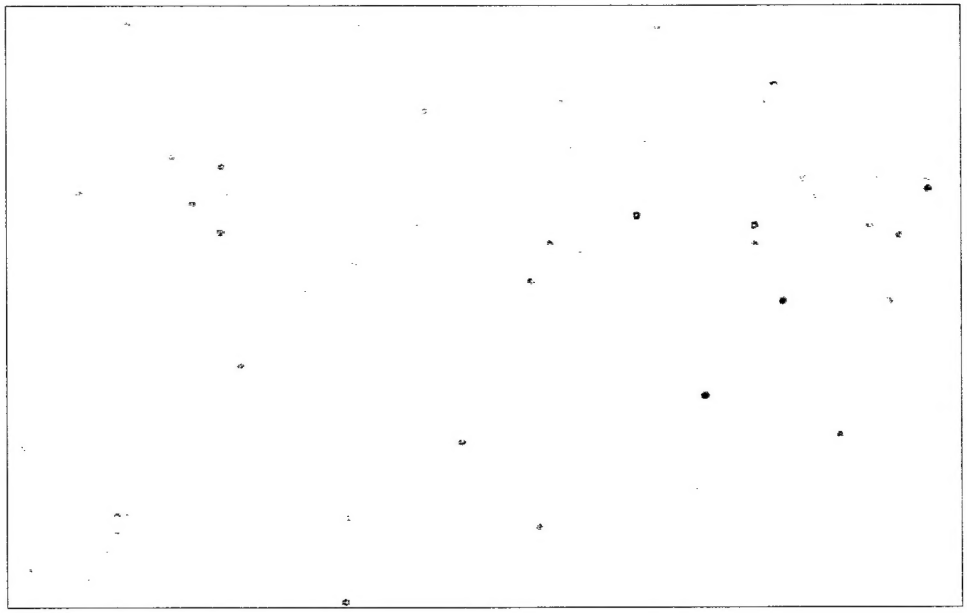


SMART cDNA



1st Round aRNA



2nd Round aRNA

Figure 6. Patterns of hybridization produced by 100 ng source RNA, cDNA produced by the SMART™cDNA synthesis kit (Clontech), or 1st or 2nd round aRNA produced by the template switching method.

Key Research Accomplishments

1. Development of a method for mapping vessel density in different areas of a solid tumor.
2. Development of a method for measuring the degree of skew which is introduced by particular amplification methods for mRNA.

Reportable Outcomes

1. Abstract at the "Era of Hope" meeting in Atlanta, GA, June 9-12, 2000 (appended).
2. Funding has been obtained to pursue the issue of microdissection and subsequent amplification of mRNA populations from the US Army Breast Cancer Program ("cDNA Libraries from Microdissected Cells in Pathological Sections", funding to begin 9/1/00).

FINAL REPORT

Personnel Receiving Support from this Research Effort:

Sandra W. McLeskey, PhD
Susette Mueller, PhD
Phyllis Vezza, MD
Arleen Emanuels, PhD

Publications/Abstracts:

1. Obtaining long amplified RNA from archival pathological sections. S.W. McLeskey[1] and J. H. Eberwine, ERA of Hope, US Army Breast Cancer Program, Atlanta, GA, June 9-12, 2000.
2. Long amplified RNA from archival paraffin-embedded pathological sections, S.W. McLeskey, A.G. Emanuels, R.S. Hannum, and J.Eberwine, manuscript in preparation.

Conclusion

Scientific conclusions to date:

1. Microvascular hot spots can be quantitated and roughly correlate with the degree of metastasis in this tumor system.

2. Current methods of amplification of mRNA produce skewed representation of individual species when compared with their representation in source RNA.

Discussion:

As mentioned in previous reports, this is very risky research. Great care must be taken to diminish the likelihood of obtaining results which are artifacts of amplification or other techniques used in cDNA synthesis. In addition, our hypothesis that gene expression in the vicinity of hot spots

is different and functional in the process of metastasis may be incorrect. However, as mentioned in previous reports, we are making methodologic advances which may be of benefit to the scientific community at large, even if this project does not succeed.

LIST OF ABBREVIATIONS AND ACRONYMS

aRNA	amplified ribonucleic acid
AFLP	amplified fragment-length polymorphism
cDNA	copy DNA
DNA	deoxyribonucleic acid
LCM	laser capture microdissection
MCF-7	an estrogen-dependent breast carcinoma cell line.
MVD	microvessel density
PCR	polymerase chain reaction
PECAM-1	platelet-endothelial cell adhesion molecule 1
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcription followed by the polymerase chain reaction
T7	bacteriophage T7

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APPENDIX

OBTAINING LONG AMPLIFIED RNA FROM ARCHIVAL PATHOLOGICAL SECTIONS

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Archival tissue specimens are valuable resources for molecular study of breast cancer and other diseases since data concerning outcomes may be associated with such specimens. The large majority of archival tissue samples are available as formalin-fixed, paraffin-embedded sections. Although microdissection is now an established technique for genetic analysis of particular cell types in archival material, analysis of gene expression in such samples has been hampered by fragmentation of extracted mRNA. However, evidence from the literature indicates that the mRNA in such sections may be intact. It is possible that covalent modifications resulting from fixation promote fragmentation during extraction or prevent effective reverse transcription. These problems might be avoided if the covalent modifications were removed and the first strand cDNA were synthesized *in situ*, directly on the section. Moreover, recent advances in cDNA synthesis have permitted attachment of manufactured sequences to the 3' end of the first strand, in a technique referred to as "template switching".

Using a combination of *in situ* reverse transcription with template switching, we have produced first strand cDNA with a T7 RNA polymerase promoter appended to its 3' end. Following harvest of the first strand from the section, second strand cDNA is synthesized. The double-stranded cDNA is then incubated with T7 RNA polymerase to produce sense-oriented amplified RNA. We have produced amplified RNA populations with a maximum length of about 7.5 kb from formalin-fixed, paraffin-embedded pathological sections of xenograft mouse tumors produced in our laboratory. Similar maximum lengths of amplified RNA populations were also obtained from random archival formalin-fixed, paraffin-embedded breast cancer sections obtained from the Lombardi Cancer Center Histophathology and Tissue Shared Resource. Yield and maximum length of amplified RNA populations may be improved somewhat by heating slides in Tris-EDTA buffer prior to *in situ* reverse transcription. This maneuver has been reported to remove covalent modifications produced by formalin fixation. Application of these techniques to single-cell microdissection by micromanipulators or by laser capture is currently being explored.

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